



PATENT  
Docket No. 367592000100

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Brian H. JOHNSTON et al.

Serial No.: 09/137,059

Filing Date: August 20, 1998

For: NUCLEIC ACID AGENTS FOR  
DETECTING TARGET MOLECULES  
AND METHODS FOR THEIR USE

Examiner: M. Schmidt

Group Art Unit: 1635

DECLARATION OF BRIAN H. JOHNSTON  
PURSUANT TO 37 C.F.R./1.132

Assistant Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

I, Brian H. Johnston, Ph.D., declare as follows:

1. I am a co-inventor of the above-referenced patent application, and am familiar with the contents and prosecution thereof.

2. I am president and CEO of SomaGenics, Inc. and a Consulting Associate Professor of Pediatrics at Stanford University School of Medicine. I received my Ph.D. in

biophysical chemistry from University of California, Berkeley. My research expertise includes the fields of nucleic acid chemistry and ribozyme technology.

3. I have reviewed the Office Action dated December 22, 2002, wherein the currently-pending claims have been rejected as allegedly not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention.

4. The following experiments were conducted under the direction of myself and Sergei Kazakov, another co-inventor of this application, in accordance with the teachings of the specification. Experiments were performed to demonstrate that the methods and RNA sequences disclosed in the specification may be used to construct a catalytically inactive RNA molecule that becomes catalytically active upon binding to a target molecule, allowing catalytic action upon a substrate other than the target molecule, and may thus be used to permit detection of the presence of the target molecule as claimed.

5. A catalytically inactive RNA molecule was constructed as shown in Figure 1. The construct depicted in Figure 1 included hairpin ribozyme core sequences as depicted in Figure 29 of the specification (domain E). For comparison, the hairpin ribozyme consensus sequence is depicted in Figure 2 of this declaration, alongside the constructs depicted in Figure 29 of the specification and the construct depicted in Figure 1 of this declaration. Both the construct shown in Figure 1 of this declaration and the construct depicted in Figure 29 of the specification include hairpin ribozyme core sequences.

The ribozyme construct depicted in Figure 1 is catalytically inactive due to the presence of a sequence that inhibits catalytic activity by internal base pairing. The catalytically inactive RNA molecule of Figure 1 was contacted with a target molecule, a fragment of TNF mRNA which bound to the catalytic RNA, displacing the internally paired sequence and permitting catalysis to occur. Constructs were prepared with internally-paired sequences of 5 to 10 base pairs in length, to optimize the length of this sequence required to make catalysis of the substrate dependent on target binding.

Upon contacting the catalytically inactive RNA molecule with the target, the ribozyme adopted a catalytically active conformation, allowing catalytic action upon non-target substrate sequences, *i.e.*, cleavage and ligation of ribozyme sequences linked in *cis* to the target binding site. In this experiment, the substrate is physically linked to the ribozyme polynucleotide. The

mechanism for target-dependent catalysis in this experiment is depicted schematically in Figure 3.

Target-dependent catalysis was detected by gel electrophoresis, as shown in Figure 4. Trace amounts of internally  $^{32}\text{P}$ -labeled ribozyme constructs of Figure 1 were incubated in 10 mM  $\text{MgCl}_2$ , 50 mM Tris-Cl, pH 8, for a total of 120 minutes at  $37^\circ\text{C}$ , either without target (lanes 1) or with 0.4  $\mu\text{M}$  non-radioactive target (a 20 nucleotide fragment of  $\text{TNF}\alpha$  mRNA which is the specific target for this construct). Samples were analyzed by denaturing 6% polyacrylamide gel electrophoresis. As depicted in Figure 3, the 5' end of the ribozyme construct used for this experiment is spontaneously self-cleaved in the absence of target. This is observed on the gel in Figure 4 as the band designated 5P. Target-dependent catalysis of the substrate sequences results in processing of the ribozyme construct to the linear (L) form. The inhibition of target-dependent substrate cleavage, *i.e.*, catalytic inactivity towards the substrate sequences in the absence of target, increased as the length of the internal pairing element increased, as shown by the increase in predominance of the 5P form over catalytically cleaved linear L form in lanes 1 as the number of internal base paired nucleotides increased from 5 to 10. In the presence of target (lanes 2), an increase in amount of the catalytically cleaved L form of the construct was observed relative to the amount of 5P form as the number of internal base paired nucleotides increased, indicating absence of catalytic activity towards the substrate in the absence of target.

These results indicate conversion from a form of the ribozyme that is catalytically inactive towards the substrate sequences to one that is catalytically active towards the substrate when the target molecule is present in the reaction mixture. Upon activation by target binding, ribozyme causes cleavage of the substrate sequences to form the L form of the RNA construct. Thus, this method may be used for detection of a target as claimed, with appearance of the L form on a gel indicative of the presence of the target.

6. Another experiment was performed to demonstrate target-dependent catalysis as claimed in the patent application. A catalytically inactive RNA molecule was constructed as shown in Figure 5A. This construct also included hairpin ribozyme core sequences as depicted in Figure 29 of the patent application and Figure 2 of this declaration, and additionally included two internal inhibitory elements that rendered the ribozyme catalytically inactive in the absence of target. Addition of the target displaced the internal inhibitory sequences, rendering the RNA

molecule catalytically active and permitting cleavage of non-target substrate sequences on the RNA molecule. This process is shown schematically in Figure 5A.

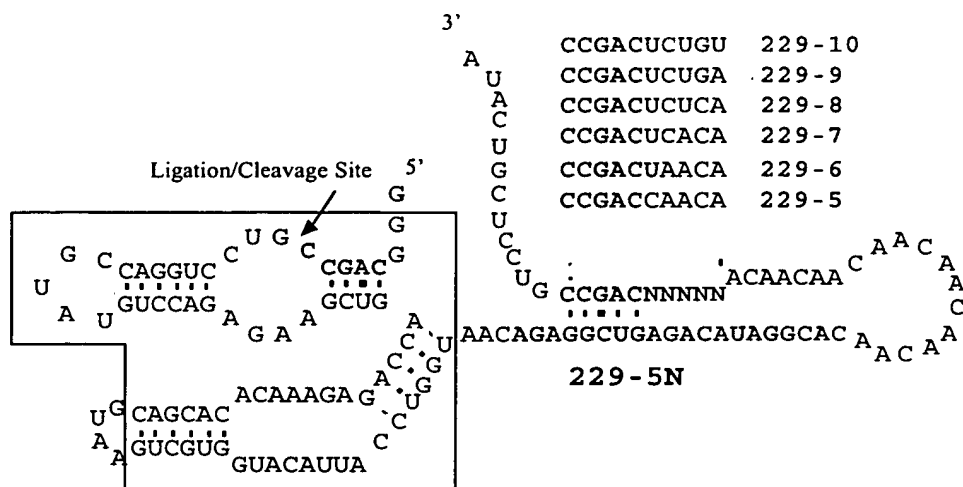
The cleavage products were detected by gel electrophoresis, shown in Figure 5B. Internally  $^{32}\text{P}$  labeled RNA constructs and target were incubated in 40 mM NaCl, 10 mM Tris-HCl, pH 7.2 at 85°C for 2 minutes and slowly cooled to room temperature. 20 mM  $\text{Mg}^{2+}$  in 50 mM Tris HCl, pH 7.2 was added to initiate the catalytic reaction. Samples were analyzed by denaturing 8% polyacrylamide gel electrophoresis. After addition of  $\text{Mg}^{2+}$ , increasing amounts of the catalytically cleaved linear L and catalytically ligated circular C forms of the construct were observed over time in reaction mixtures that contained 100  $\mu\text{M}$  target (lanes 2-7), as well as 45 and 50 nt cleavage products. Further, with a constant reaction time of 20 minutes and varying target concentrations, increasing amounts of the L and C forms were observed as the target concentration increased. In absence of target (lane 8), these products were not observed, indicating that catalysis was dependent on target binding.

This scheme may be used for detection of a target molecule as claimed in the patent application because the L and C products of catalytic action of the ribozyme on the substrate sequences are observed only in the presence of target.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

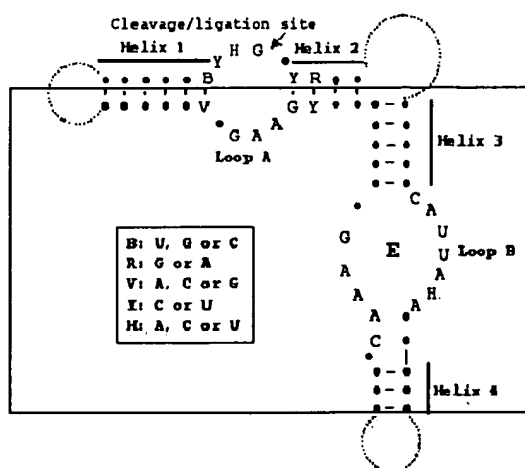
\*\*\*\*July 21, 2003\*\*\*\*  
Date

  
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Brian H. Johnston, Ph.D.



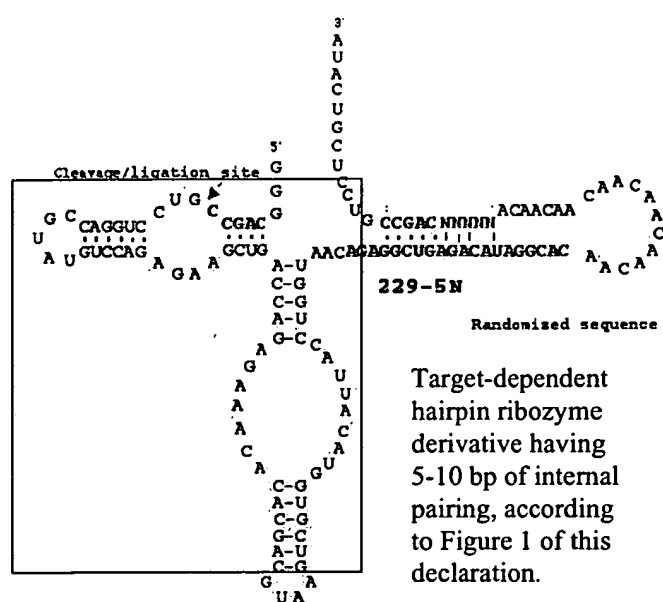
**Figure 1. Structure of allosterically regulated cis-cleaving and self-ligating derivatives of the hairpin ribozyme in their unprocessed form, designated ALR229-5, 229-6, 229-7, 229-8, 229-9 and 229-10.** These molecules differ in the length of their internal pairing, having respectively 5, 6, 7, 8, 9 and 10 bp. The cleavage and ligation site is denoted by an arrowhead. 5' processing occurs spontaneously, whereas 3' processing requires binding of the target to displace the internal base-pairing (see Fig. 3). The boxed region is the hairpin ribozyme core sequences (see Fig. 2C).

A

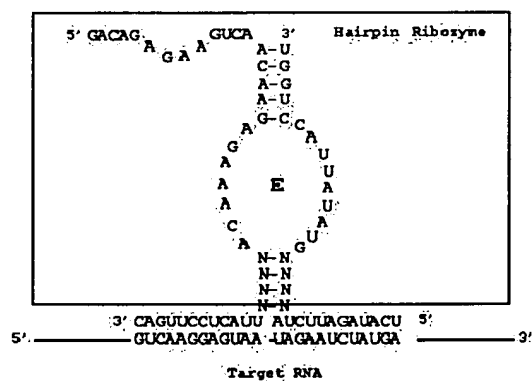


Core hairpin ribozyme structure and consensus sequence, circular form

C

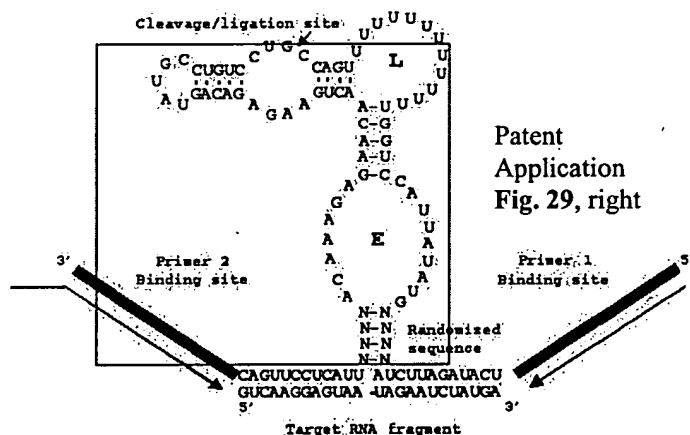


B

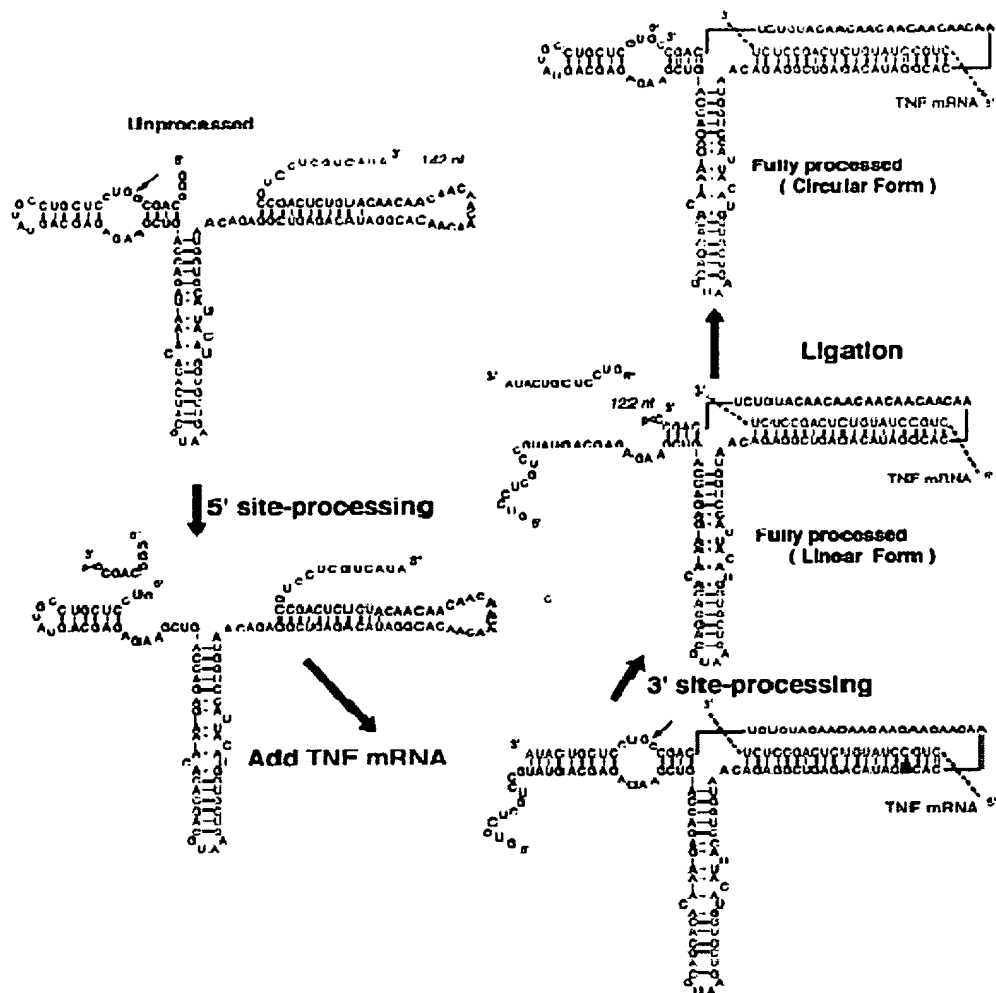


Patent application Fig. 29, left

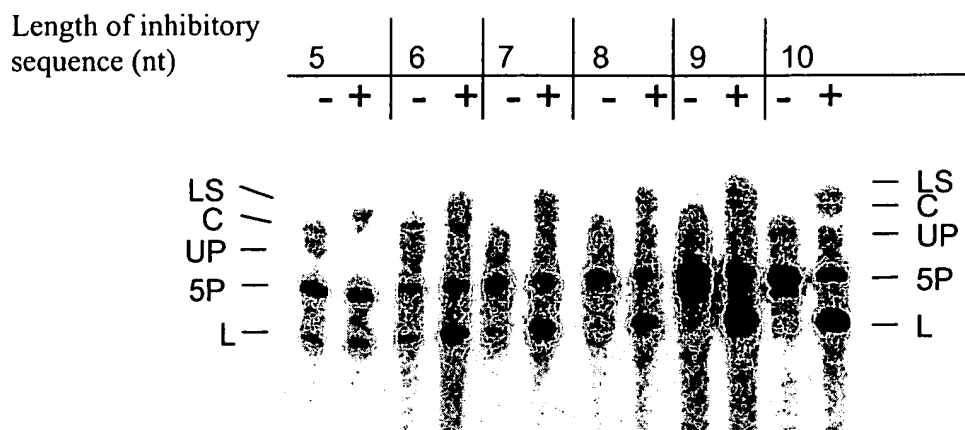
D



**Figure 2.** Comparison of the core hairpin ribozyme consensus sequence (A) and the catalytic domain and substrate sequences of the constructs described in Figure 1 of this declaration (C) with the constructs shown in Figure 29 of the Patent Application (B and D, respectively). Boxed areas in A and C are nearly identical to those in B and D except in double-helical regions, where actual sequence is irrelevant as shown by the dots in the consensus structure (A).

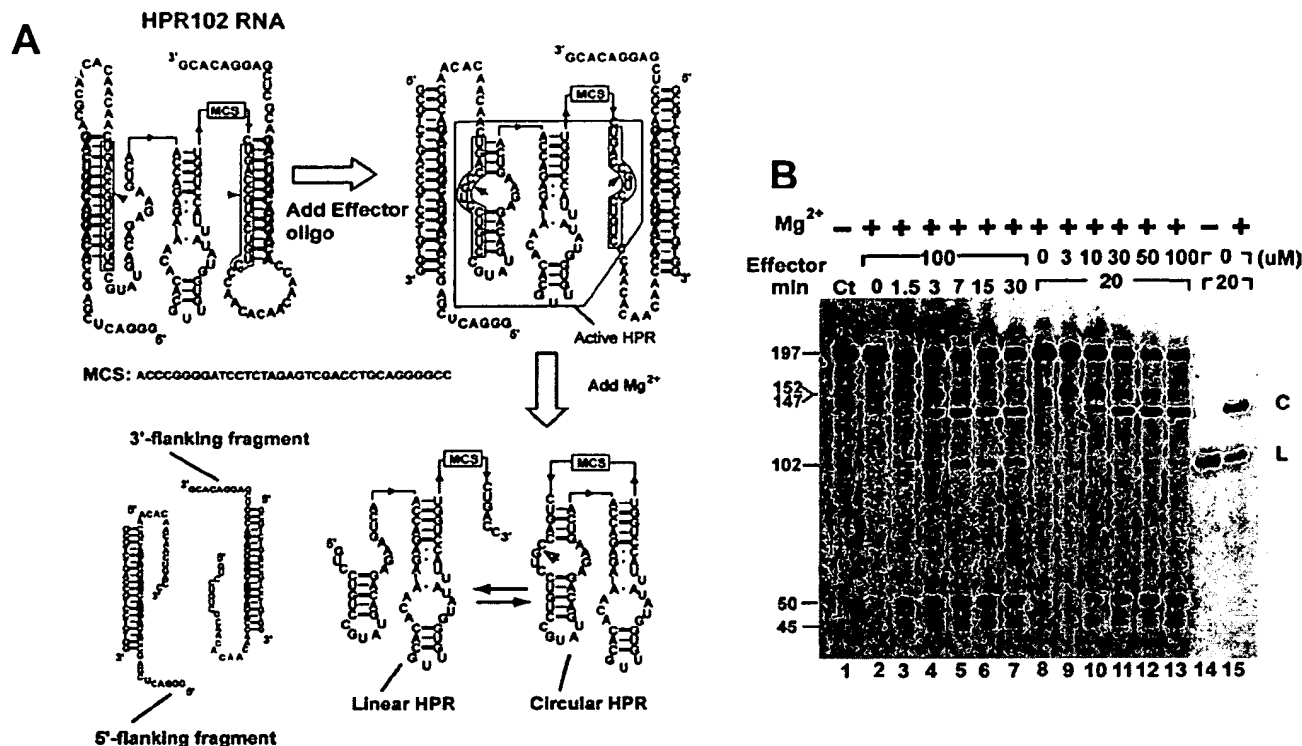


**Figure 3. Scheme of target-dependent and target-independent self-processing of ribozyme 229-10 depicted in Figure 1.** The unprocessed molecule undergoes self-cleavage at the 5'-end. The processing of the 3'-end is inhibited by its base pairing with the antisense sequence. Upon binding to the target, this base pairing is disrupted the 3' end can bind to the substrate binding site, displacing the already-processed 5' end, allowing 3'-end processing to proceed. The fully processed ribozyme, bound to the target, can then undergo circularization.



**Figure 4. Binding of latent ribozymes ALR229-5 through ALR229-10 with TNF-20, a 20-nt fragment of the target TNF $\alpha$  RNA, reveals the target-dependent activation of ribozyme self-processing.** Trace amounts of internally  $^{32}\text{P}$ -labeled latent ribozymes were incubated in 10 mM  $\text{MgCl}_2$  / 50 mM Tris-Cl (pH 8) for a total of 120 minutes at 37°C, either without (-) or with (+) non-radioactive 0.4  $\mu\text{M}$  TNF-20 target (lanes 2). Samples were analyzed by denaturing 6% polyacrylamide gel electrophoresis. Abbreviations: LS, complex with target; C, circular forms of fully-processed molecules; UP, unprocessed RNA transcripts; 5P, 5'-end semi-processed molecules; L, fully-processed linear molecules.





**Figure 5: Cleavage and ligation of effector-activated hairpin ribozyme (HPR).**

**A.** Scheme for effector-dependent processing of HPR-102 RNA induced by the effector/target oligonucleotide HPR102-3. HPR102 RNA (197 nt) contains two self-inhibition sequences, two substrate sequences, latent hairpin ribozyme catalytic core, and linker sequence encoding a multiple cloning site (MCS).

**B.** Effector-dependent processing of HPR-102 RNA. HPR102 RNA (internally <sup>32</sup>P-labeled) and the 19-nt effector (target) oligonucleotide (0-100 μM) in TN buffer (40 mM NaCl, 10 mM Tris-HCl [pH 7.2 at 45°C]) were heated to 85°C for 2 min and slowly cooled to room temperature. 20 mM Mg<sup>2+</sup> and 50 mM Tris-HCl (pH 7.2) were then added to initiate the self-processing of HPR-102 RNA and the mixture was incubated at 45°C for the times shown. Self-cleavage at one of the substrate cleavage sites gave 152+45 nt products; cleavage at the other gave 147+50 nt products. Cleavage at both sites generated the linear hairpin ribozyme (HPR, 102 nt, designated L), which remained active and could circularize (C) by ligating its own 5' and 3' ends. Increasing yields of fully processed C and L as well as the 45- and 50-nt ends are seen with increasing time (lanes 2-7) and concentration of effector (lanes 8-13). As markers, a linear HPR (lane 14) was purified from the transcription of a control RNA (M101) that contained the same hairpin ribozyme core sequence, but without the inhibitory sequences. In the presence of Mg<sup>2+</sup> (20 mM), the circular HPR (lane 15, C) appeared with a slower mobility on a denaturing 8% polyacrylamide gel.